

# Evolution of Receptors for Growth Hormone and Somatolactin in Fish and Land Vertebrates: Lessons from the Lungfish and Sturgeon Orthologues

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**Abstract** Two cognate hormones, growth hormone (GH) and somatolactin (SL), control several important physiological processes in vertebrates. Knowledge about GH and its receptor (GHR) has accumulated over the last decades. However, much less is known about SL and its receptor (SLR). SL is found only in fish (including lungfish), suggesting that it was present in the common ancestor of vertebrates, but was lost secondarily in the lineage leading to land vertebrates after the lungfish branched off. *SLR* was suggested to be a duplicated copy of *GHR* acquired only in teleosts via the fish-specific genome duplication (FSGD). This scenario (i.e., the existence of SL but not SLR in the vertebrate ancestors) is intriguing but contested. In this study, we first evaluated the plausibility of this scenario through synteny analyses and found that the loci for *GHR* and *SLR* are located in syntenic genomic positions, whereas the loci for *GH* and *SL* are not. Next, we cloned *GHR*s of lungfish and sturgeon, which possess SL but did not undergo the FSGD (i.e., they should not possess SLR). Their phylogenetic positions in the *GHR/SLR* gene tree further support the fish-specific scenario for the GHR SLR duplication. Interestingly, their sequences share greater similarity with teleost SLRs and reptilian/amphibian GHRs than with the GHRs of mammals, birds, and teleosts. On the basis of these results, we discuss the validity of the nomenclature of the teleost-specific copy of GHR as SLR and an ancestral receptor(s) for SL before the evolution of SLR during the FSGD.

**Keywords** Somatolactin receptor (SLR) · Growth hormone receptor (GHR) · Lungfish · Sturgeon · Fish-specific genome duplication (FSGD) · Synteny · Sub-functionalization

## Introduction

Somatolactin (SL) is a hormone that belongs to the growth hormone (GH)/prolactin (PRL) family, and is either up- or down-regulated during various important physiological activities in fish (Fukada et al. 2005 and references therein). Although direct evidence for the role of SL in these activities is mostly lacking, the recent identification of a SL-deficient mutant in medaka (*color interfere, ci*) demonstrated that SL functions in body-color regulation, lipid metabolism, or cortisol secretion in vivo (Fukamachi et al. 2004, 2005, 2006). Further analyses of the *ci* phenotypes might provide evidence for other potential functions since the SL receptor (SLR) is rather broadly expressed in various organs (Fukada et al. 2005; Saera-Vila et al. 2005; Fukamachi et al. 2005; Jiao et al. 2006; Ozaki et al. 2006).

The current nomenclature of the fish SLR is controversial and confusing, as is often the case with gene families that resulted from complicated gene duplications (Mindell and Meyer 2001). The confusion arose because *SLR*, which was first defined in salmon (Fukada et al. 2005), is orthologous to a subset of growth hormone receptors (*GHR*s, particularly *GHR1*) of other teleosts (Fukamachi et al. 2005). Several teleost species have two divergent *GHR*-like genes that exhibit distinctive residual and structural differences, and whose sequences are less than 40% identical. In addition, their in vitro characteristics were shown to be different in salmon (Fukada et al. 2004, 2005);

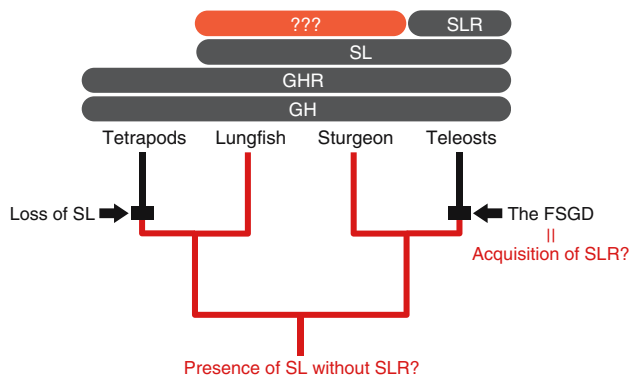
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i.e., one of the GHRs binds GH, while the other predominantly binds SL (in this manuscript, we call the former GHR and the latter SLR). Nevertheless, the teleost *GHR* and *SLR* are often assumed to be duplicated copies of an ancestral *GHR* (Saera-Vila et al. 2005; Jiao et al. 2006; Ozaki et al. 2006) that were acquired through the fish-specific genome duplication (FSGD).

The FSGD, which occurred in the lineage leading to modern (teleost) fishes (Hoegg and Meyer 2005; Meyer and Van de Peer 2005), produced many teleost-specific duplicated genes (Meyer and Schartl 1999; Postlethwait et al. 2004; Mitani et al. 2006; and references therein). However, the evolutionary scenario, that *SLR* is a teleost-specific paralogue of *GHR*, remains an open question. This is because lungfish and sturgeon, which did not experience the FSGD, and hence should not possess duplicated copies of *GHR* (i.e., *SLR*), indeed do have *SL* (Amemiya et al. 1999; May et al. 1999). These findings suggest that the *SL* gene already existed in the common ancestor of vertebrates, but that *SLR* was later acquired via the FSGD only in teleosts (Fig. 1).

In this study, we used available synteny data to first assess whether or not the teleost *SLR* is really a duplicated copy of *GHR*. We then cloned *GHR*s of lungfish and sturgeon. Because both of these fish are expected to possess the ancestral hormone receptor set (i.e., GH, SL, and one copy of GHR), which is different from that of tetrapods (i.e., GH and one copy of GHR) or teleosts (i.e., GH, SL, and two copies of GHR), their *GHR* sequences might have unique features, and could be helpful for characterizing the evolutionary history of the receptor(s) for SL.



**Fig. 1** The hypothesis addressed in this study. Phylogenetic relationships among tetrapods, lungfish, sturgeon, and teleosts are shown. GH exists in all lineages. GHR has been identified in tetrapods and teleosts. SL has been identified so far only in the fish lineages, indicating that SL should have been secondarily lost from land vertebrates (arrow on the left). To date, SLR has been found only in teleosts and may be a duplicated copy of GHR acquired via the FSGD (arrow on the right). This evolutionary scenario, however, assumes a hormone receptor set of GH, SL, and GHR, but not SLR in some fish lineages and the common vertebrate ancestor (indicated by red lines)

## Materials and Methods

### Synteny

Genomic sequences containing *GHR*, *SLR*, *GH*, or *SL*, were retrieved from the following databases: National Center for Biotechnology Information (NCBI; human, <http://www.ncbi.nlm.nih.gov/genome/guide/human/>), University of Tokyo Genome Browser (UTGB; medaka, <http://www.medaka.utgenome.org/>, version 1.0), Joint Genome Institute (JGI; fugu, <http://www.genome.jgi-psf.org/Takru4/Takru4.home.html>, version 4.0), and Ensembl (zebrafish, [http://www.ensembl.org/Danio rerio/index.html](http://www.ensembl.org/Danio%20rerio/index.html), version 6.0). Annotation of human chromosomes followed that of the NCBI database. We manually reannotated other teleost sequences by blastx, and the human gene with the lowest e-value was used for the annotation. In cases where multiple human genes showed similarly low e-values (e.g., genes in a gene family), all were included. We ignored any transposase-like or reverse transcriptase-like sequences, which represented repetitive sequences.

### Fish

We used lungfish (*Protopterus dolloi*) and sturgeon (*Huso dauricus*) samples stored at  $-80^{\circ}\text{C}$  in the laboratory. We confirmed the species' identity by sequencing fragments of their 16S rRNA using universal primers (5'-CGCCTG TTTAACAAAAACAT-3' and 5'-CCGGTCTGAACTCA GATCACGT-3') and constructing phylogenetic trees with corresponding sequences available on the GenBank database (data not shown).

### Cloning

We designed four degenerate primers for nested reverse-transcriptase polymerase chain reactions (RT-PCR) on the regions relatively conserved among vertebrate GHR/SLRs. Primer sequences for the first PCR are f: 5'-ATH-GTNCARCCNGAYCCNCC-3' and r: 5'-ARYTCDATRA AYTCNACCCA-3', and those for the second PCR are f: 5'-YTNAAYTGGACNYTNYTNA-3' and r: 5'-TCDATNC CYTTDATYTTNGG-3'. Total RNA was extracted from frozen muscle samples using TRIZOL (Invitrogen), and template cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) using 3' (5'-ATTCTAGAGGCC GAGGCGGCCGACATGTTTTTTTTTTTTTTTTTTTTVN-3') and 5' (5'-AAGCAGTGGTATCAACGCAGAGTGGCCA TTATGGCCGGG-3') adaptors. RT-PCR parameters were  $94^{\circ}\text{C}$  for 1 min; 40 cycles of  $98^{\circ}\text{C}$  for 20 s,  $40^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 min; with a final extension at  $72^{\circ}\text{C}$  for 10 min.

Amplified bands were excised from an agarose gel, purified using HiBind DNA column (Peqlab), and directly sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems).

To obtain 5' and 3' flanking regions of the cDNAs, we performed rapid amplification of cDNA ends (RACE) using 5' or 3' primers complementary to the adaptors (5'-AAGCAGTGGTATCAACGCAGAGT-3' or 5'-ATTCTA GAGCCGAGGCGGC-3', respectively) and species-specific nested primers, designed according to the cDNA sequences obtained above (data not shown). PCR parameters were 94°C for 1 min; 30 cycles of 98°C for 20 s, 60°C for 1 min, 72°C for 3 min; with a final extension at 72°C for 10 min. We used TaKaRa LA Taq polymerases (Takara) for the reactions and reproducible bands were identified, excised, and directly sequenced as described above. We designed additional species-specific primers that amplified overlapping regions of the entire cDNAs and verified that their sequences were consistent with the results from the degenerate RT PCR and RACE experiments.

### Phylogeny Reconstruction

Amino acid sequences of GHR, SLR and PRL receptor (PRLR) of vertebrates were retrieved from the GenBank and aligned by ClustalW (<http://www.ebi.ac.uk/clustalw/>). We used Phyml (Guindon and Gascuel 2003; <http://www.atgc.lirmm.fr/phyml/>) and MrBayes (Ronquist and Huelsenbeck 2003; <http://www.mrbayes.csit.fsu.edu/>) software under the JTT+I+G+F substitution model following the results from ProtTest (Abascal et al. 2005; <http://www.darwin.uvigo.es/software/prottest.html>) and ModelGenerator (Keane et al. 2006; <http://www.bioinf.may.ie/software/modelgenerator/>).

### Protein Structure Prediction

We used the SOSUIsignal program (Gomi et al. 2004; [http://www.bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal\\_submit.html](http://www.bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html)) and NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) to predict N-terminal signal peptides, transmembrane regions, and potential N-glycosylation sites. To predict secondary structures, we used the New Joint method (Ito et al. 1997; [http://www.cbrc.jp/papia/cgi/ssp\\_query.pl?query=seq](http://www.cbrc.jp/papia/cgi/ssp_query.pl?query=seq)). As the New Joint server does not allow protein sequences exceeding 500 amino acids as a query, we manually trimmed excess C-terminal amino acids before submitting sequences. Since it also does not permit a sequence with

undetermined amino acid (X) as a query, three Xs contained within signal peptides of the opossum GHR (NM 001032976) had to be removed prior to this analysis. For the same reason, we could not use coho salmon GHR1 (AF403539), since it contains an X in the middle of the extracellular domain.

### Genomic Structure

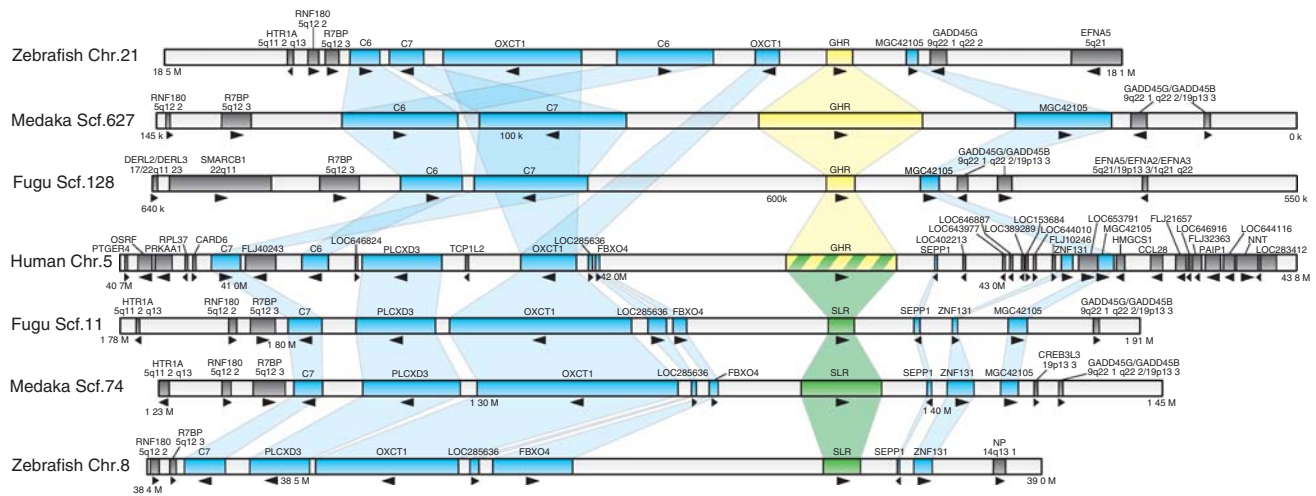
Genomic DNA of lungfish and sturgeon were extracted from muscle tissue by phenol-chloroform extraction. Genomic DNA of eel (*Anguilla japonica*) was kindly provided from elsewhere (see the "Acknowledgements"). Species-specific primers were designed to sandwich the region where the *SLR*-specific intron presumably exists, and were used for both PCR with template genomic DNA and RT-PCR. Products were run on 1% agarose gels, stained by ethidium bromide, photographed on an ultraviolet (UV) transilluminator, and then sequenced.

## Results

### *SLR* is a Teleost-Specific Parologue of *GHR*, Whereas *SL* is Not a Teleost-Specific Parologue of *GH*

By taking advantage of the available whole or draft genome sequences, we investigated genes surrounding the *GHR* and *SLR* loci of vertebrates to determine if synteny is conserved. Using a medaka *GHR* sequence (GenBank accession number DQ010539) on scaffold (Scf) 627, we found (by tblastx) that fugu and zebrafish have orthologues on Scf 128 and chromosome (Chr) 21, respectively. Using the medaka *SLR* sequence (DQ002886) on Scf 74, we found orthologues on Scf 11 of fugu and Chr 8 of zebrafish. All of these scaffolds/chromosomes contain sequences similar to human genes located on Chr 5p12 13.1 (Fig. 2). This well-conserved synteny suggests that not only the *GHR*s, but also the *SLR*s of medaka, fugu, and zebrafish, are orthologous to human *GHR*. Therefore, the teleost *SLR* was likely acquired through a duplication of *GHR*. Considering that this duplication involved a rather large chromosomal region (note that synteny of > 2 Mb of the human chromosome is conserved), the FSGD would most likely be the cause of this *GHR* duplication (Fig. 4).

We similarly analyzed synteny around the *SL* and *GH* loci. *GH*s of fugu (U63807), medaka (AF134606), and zebrafish (AJ937858) are located on Scf 7980, Scf 697, and Chr 3, respectively. Although the fugu scaffold was not sufficiently long for this analysis, both the medaka and zebrafish *GH* loci showed conserved synteny with the human *GH* cluster locus located on Chr 17q23-25 (Fig. 3).



**Fig. 2** The conserved synteny between the *GHR* and *SLR* loci. Horizontal bars represent partial scaffolds/chromosomes whose positions are indicated below (bp). Species names and scaffold/chromosome numbers are indicated on the left. Colored boxes in the bars indicate genes we manually annotated (see the “Materials and Methods” section); yellow, *GHR*; green, *SLR*; blue, syntenic; and grey, nonsyntenic genes. Gene names and their locations on human

chromosomes (if they are nonsyntenic) are indicated on top. Arrowheads indicate 5' 3' orientations of each gene. Note that multiple genes surrounding the human *GHR* locus (*C7*, *C6*, *PLCXD3*, *OXCT1*, *LOC285636*, *FBXO4*, *SEPP1*, *ZNF131*, and *MGC42105*) are found in similar positions/orientations around both the *GHR* and *SLR* loci of these teleost genomes

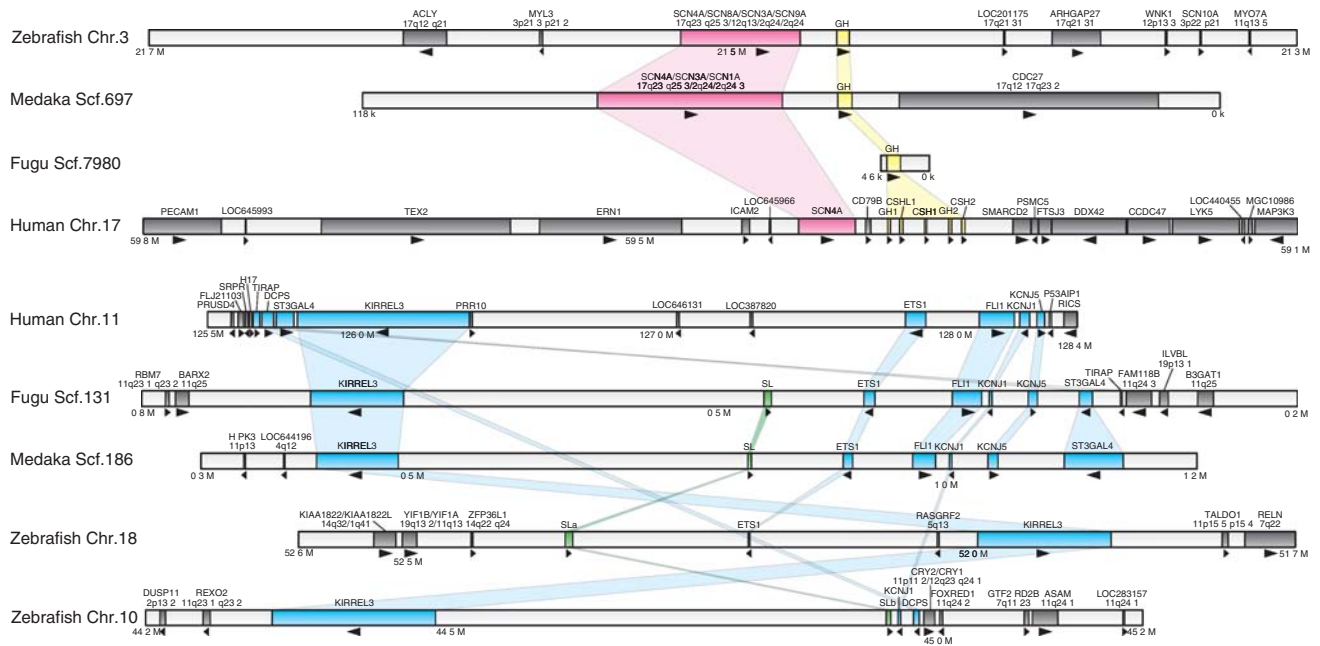
Medaka *SL* (AY530202) and its fugu orthologue (predicted) are located on Scf 186 and Scf 131, respectively. Zebrafish *SL* (*SLa*; AY376857) is located on Chr 18. Their flanking regions commonly contain sequences similar to human genes on Chr 11q24 (instead of Chr 17q23-25), where no apparent trace of *SL* can be identified (a missing orthologue; Postlethwait 2006). Importantly, another copy of zebrafish *SL* (*SLb*; AY221126) is located on Chr 10 and its flanking regions also contain sequences similar to human genes on Chr 11q24. These observations support orthology relationships within, but not between, *GHR*s and *SL*s. Therefore, unlike *SLR*, *SL* should have existed prior to the FSGD, which produced *SLa* and *SLb* paralogs in teleosts. Whereas the presence of *SLb* has been reported for some teleost species other than zebrafish (Zhu et al. 2004), we could not find *SLb* in the genomes of medaka and fugu.

#### Isolation of Lungfish and Sturgeon GHRs

The teleost-specific *GHR* duplication suggests that the hormone receptor set of GH, SL, and GHR, but not *SLR*, actually existed in the common vertebrate ancestor. For further characterization of the receptor evolution, we determined the *GHR* sequences of lungfish and sturgeon, both of which, due to their phylogenetic position, are expected to have retained this ancestral hormone receptor set (Fig. 1).

We constructed degenerate primers at regions conserved in both *GHR*s and *SLR*s of vertebrates (in order to amplify both, if they exist) and performed nested RT-PCR using muscle cDNAs as templates. Muscles are one of the organs in which both *GHR* and *SLR* are most strongly transcribed in at least some teleosts (Fukada et al. 2004, 2005; Saera-Vila et al. 2005; Fukamachi et al. 2005). Direct sequencing of the amplified products using degenerate primers resulted in homologous gene sequences in both the lungfish and sturgeon (and also *Polypterus senegalus*; data not shown), indicating that only a single copy of *GHR* was amplified, as expected. We determined entire cDNA sequences by RACE and termed them *GHR* (instead of *SLR*), according to the evolutionary considerations we have described.

The lungfish *GHR* (EF158850) consists of 602 606 amino acids (we found possible allelic polymorphisms that cause several amino acid substitutions and insertions/deletions; data not shown) and phylogenetic trees using PRL receptors (PRLRs) as an outgroup placed it basal to tetrapod *GHR*s (Fig. 4). The extracellular (hormone-binding) domain (ECD) of the lungfish *GHR* is most similar to those of tetrapod *GHR*s, with 39% (guinea pig) to 47% (pigeon) shared sequence identities, followed by teleost *SLR*s, with 35% (halibut) to 41% (eel/carps) identities, and teleost *GHR*s, with 31% (catfish) to 36% (eel) identities. It is intriguing to note that the lungfish *GHR* is always more similar to *SLR*s than to *GHR*s in all teleost species from



**Fig. 3** The lack of synteny between the *GH* and *SL* loci. Horizontal bars represent partial scaffolds/chromosomes whose positions are indicated below (bp). Species names and scaffold/chromosome numbers are indicated on the left. Colored boxes in the bars indicate genes we manually annotated; yellow, *GH*; green, *SL*; blue or pink, syntenic; and grey, nonsyntenic genes. Gene names and their locations on human chromosomes (if nonsyntenic) are indicated on

top. Arrowheads indicate 5' 3' orientations of each gene. Note that the syntenic genes around the *GH* locus (*SCN4A*) and those around the *SL* locus (*TIRAP*, *DCPS*, *STSGAL4*, *KIRREL3*, *ETS1*, *FLI1*, *KCNJ1*, and *KCNJ5*) are located on different chromosomes in human. On the other hand, conserved synteny is observed between the *SLa* and *SLb* loci of zebrafish (bottom)

which both GHR and SLR have been cloned (i.e., salmon, seabream, medaka, tilapia, catfish, and eel).

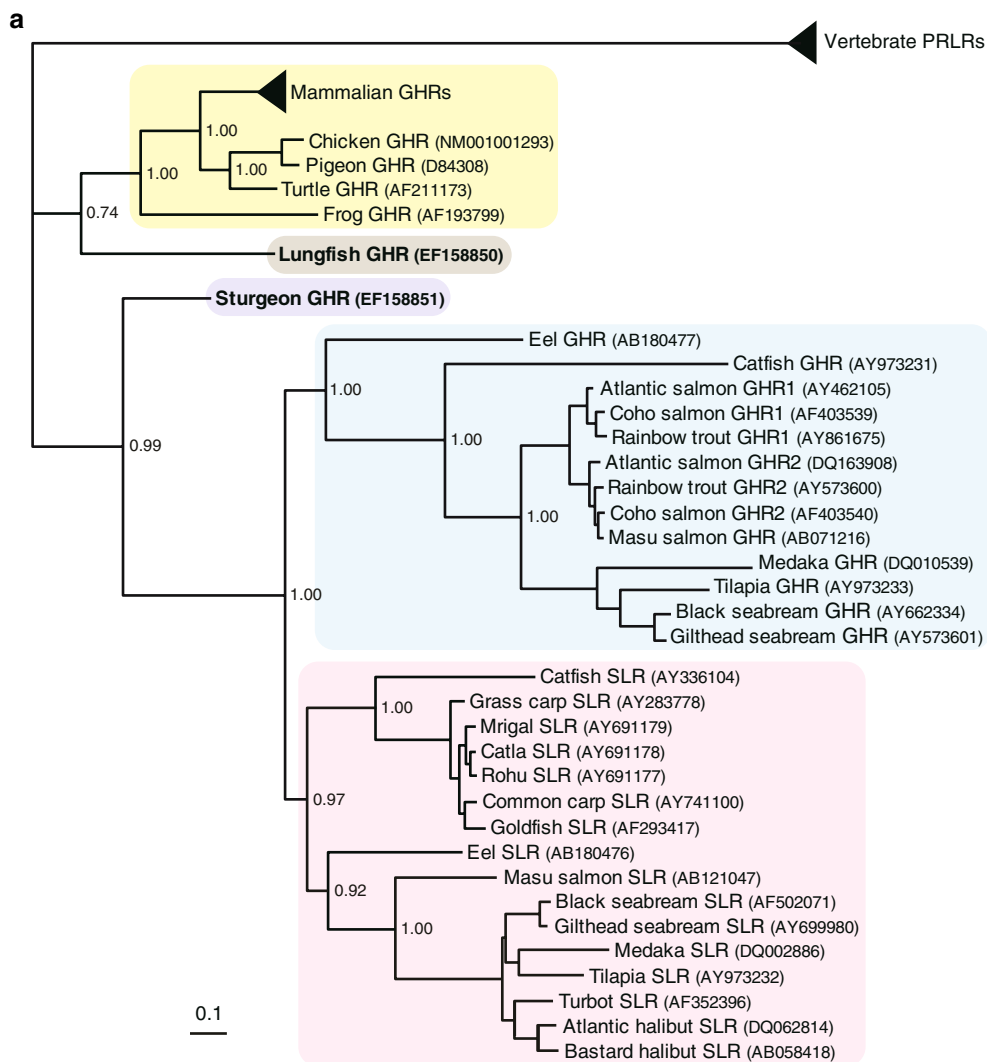
The sturgeon GHR (EF158851) consists of 571 amino acids and phylogenetic trees supported its position basal to both the GHRs and SLRs of teleosts (Fig. 4). This result further supports the hypothesis that *SLR* is a teleost-specific duplicated copy of the ancestral *GHR*. The ECD of the sturgeon GHR is most similar to those of teleost SLRs, with 39% (tilapia) to 54% (carp) identities, followed by tetrapod GHRs with 39% (buffalo/guinea pig) to 48% (turtle) identities, and teleost GHRs, with 33% (catfish) to 44% (rainbow trout) identities. The exception is eel GHR (47%), which exceptionally retained SLR-like features (see below). Again, the sturgeon GHR is always more similar to SLRs than to GHRs in all teleost species. The e-values of the blastp analyses also support that the lungfish/sturgeon GHRs are more similar to teleost SLRs than to teleost GHRs (data not shown). However, significantly more rapid diversification of GHRs than SLRs in teleosts was not always supported by the relative-rate test using the MEGA3 software (Kumar et al. 2004;  $p = 0.000$  0.216); the same test did support more rapid diversification of both teleost receptors than sturgeon GHR ( $p < 0.001$ ; data not shown; Robinson-Rechavi and Laudet 2001).

#### Lineage-Specific Features of the Vertebrate GHR/SLRs: From Their Residual Comparisons

Molecular mechanisms of the GH GHR interaction have been extensively studied using mammalian models and functionally important amino acids at the ECD have been structurally, biochemically, and genetically determined. In order to further characterize the lungfish and sturgeon GHR sequences, we compared their equivalent residues with those of other receptors (Figs. 5 and 6).

One apparent difference specifically found in teleost GHRs is the lack of two of six disulfide-bond-forming cysteines, which are perfectly conserved among tetrapod GHRs and teleost SLRs. In the eel, however, both GHR (AB180477) and SLR (AB180476) retain all six cysteines, as do the lungfish and sturgeon GHRs. Therefore, after the FSGD and the separation of eels from the other teleost lineages, two of the six ancestral cysteines were lost specifically in teleost GHRs.

The WSXWS-equivalent motif (the Y/FGXFS motif) seems conserved throughout the vertebrates (with the exception of AGEFS in the eel GHR). We found that the placental-specific Y222 also occurs in lungfish, though the phenylalanine (found in sturgeon) still seems to represent an ancestral state.



**Fig. 4** Phylogenetic trees of the vertebrate GHR/SLR/PRLRs as inferred from the (a) MrBayes and (b) PhyML software. Posterior probabilities or bootstrap values are shown at several representative nodes. Detailed subtrees of vertebrate PRLRs and mammalian GHRs were constructed but are not shown. Numbers in brackets are GenBank accession numbers

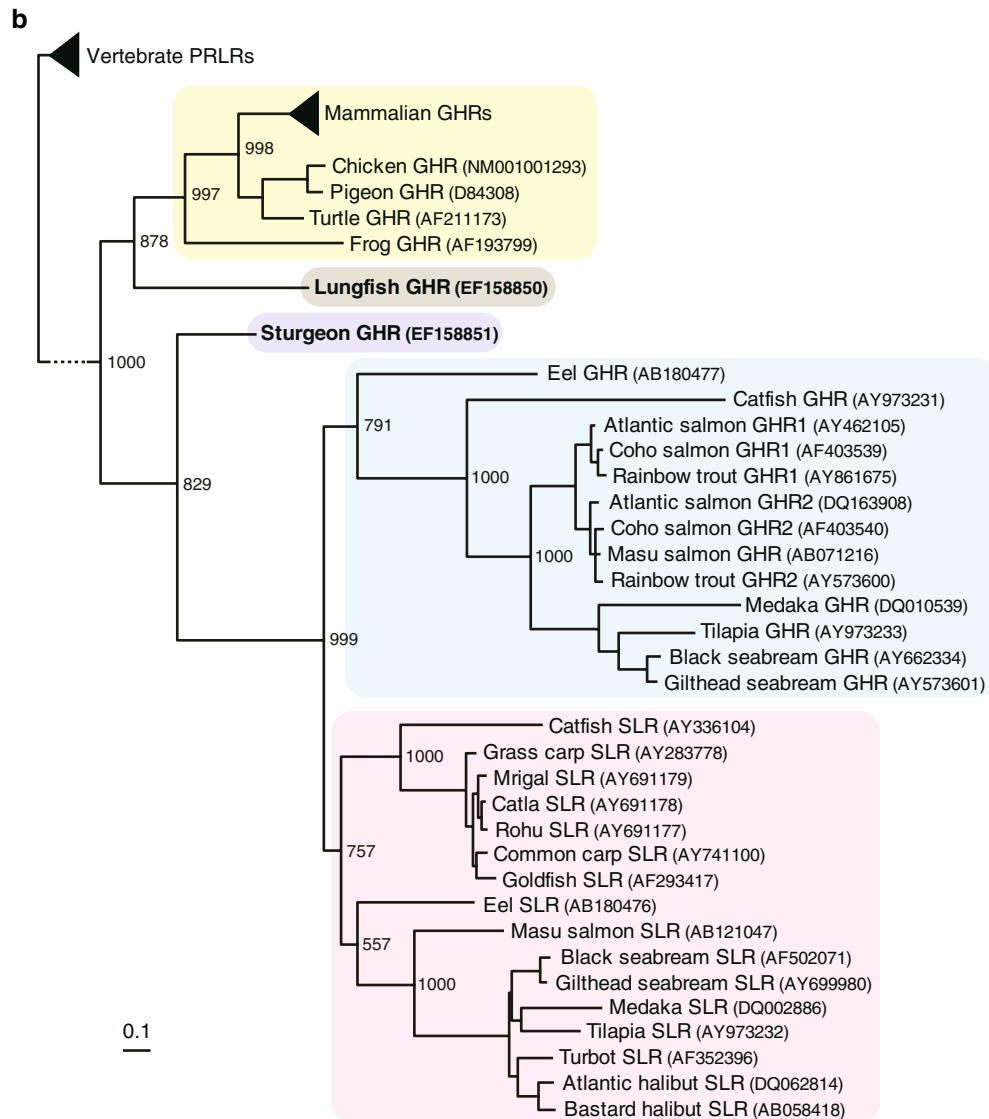
GHR dimerizes when being activated, and residues involved in the dimerization have also been identified (de Vos et al. 1992). Several mutations have also been identified from human patients with GH-insensitive dwarfism (Laron syndrome; Laron 2004), indicating that substituted residues are functional (or substituting residues are mal-functional). Some amino acids at these sites show strict conservation (e.g., F96 and P131), while others are only conserved within lineages (e.g., R161 and S201).

Asparagines N97, N138, and N182 (located after the fifth beta strand, within the eighth, and after the tenth beta strand in Fig. 6) were shown to be glycosylated and important for GH binding in pig (Harding et al. 1994). Whereas N97 is strongly conserved in all the receptors, N138 and N182 seem not to be conserved among teleost GHRs. Lungfish and sturgeon GHRs retain all the three

glycosylation sites, suggesting more derived states of teleost GHRs.

There is an unpaired cysteine (C241) in the human GHR that forms a disulfide bond during receptor dimerization (Zhang et al. 1999). Although this bond was shown to be dispensable, this cysteine is perfectly conserved among mammalian, avian, and reptilian GHRs. Equivalent cysteines are not found in amphibian GHRs, teleost SLRs, or teleost GHRs. Instead, they have an unpaired cysteine between the 13th and 14th beta strands, with the exceptions of eel SLR and catfish GHR. Like the receptors of lower vertebrates, the lungfish and sturgeon GHRs also have the unpaired cysteine between the 13th and 14th beta strands.

Of the approximately 10 structural/functional residues that actually participate in the GH GHR interaction (Harding et al. 1994; de Vos et al. 1992; Clackson & Wells



**Fig. 4** continued

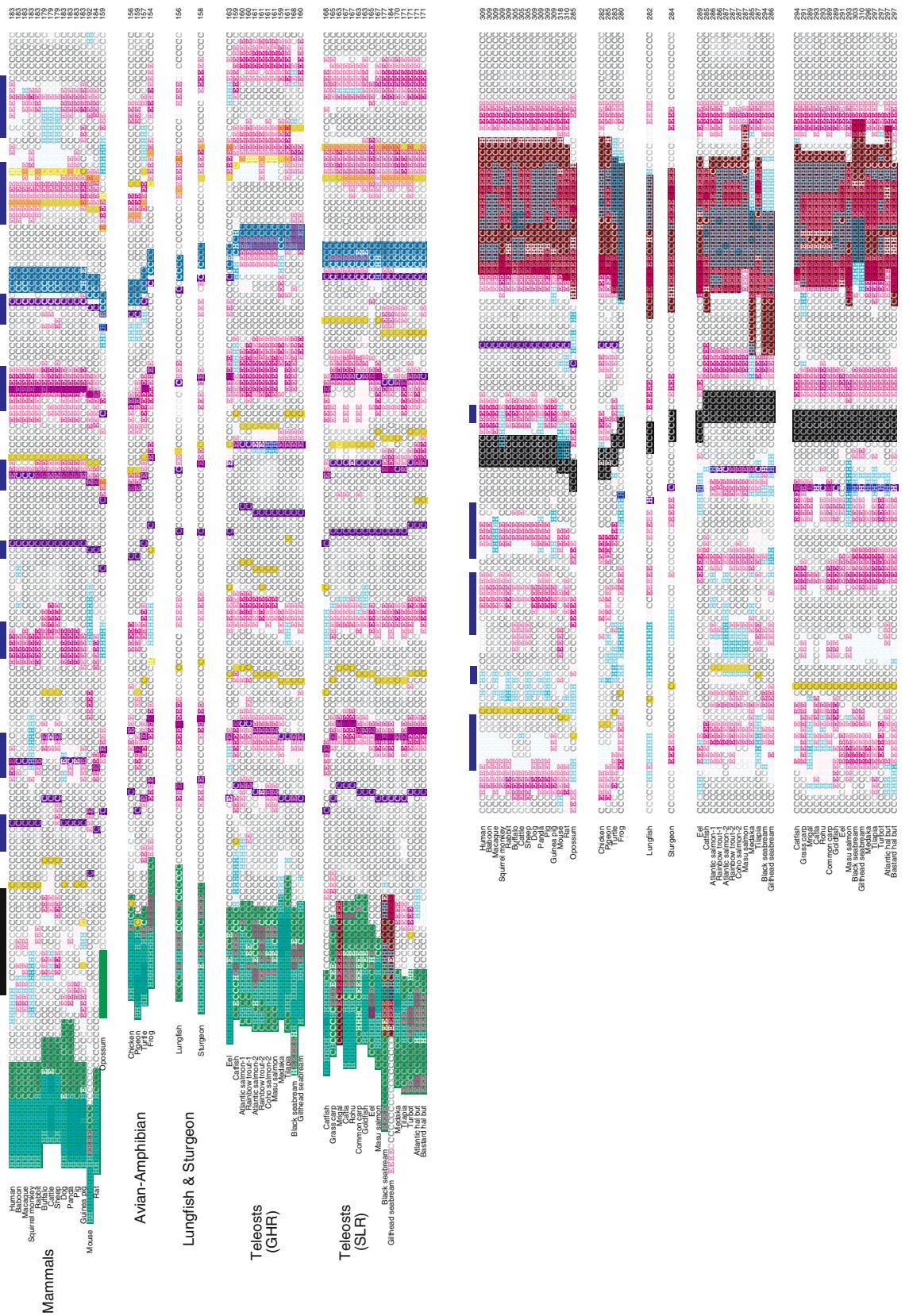
1995; Clackson et al. 1998), those that contribute the most to the binding affinity, W104 and W169, are perfectly conserved in all the GHR/SLRs. This suggests that the fundamental mechanisms for the hormone receptor binding did not diverge during the receptor evolution. D164 is also completely conserved, though it has received less attention to date. R43 is one of the most interesting residues because it determines the binding specificity of human GHR, which is restricted only to GHs of Old World primates (Souza et al. 1995; Yi et al. 2002). The arginine (positively charged) at R43 interacts with aspartic acid (negatively charged) at D171 of the primate GHs. GHs (and also SLs) of other vertebrates have histidine (positively charged) at D171, which may cause ionic repulsion against the R43. Instead of R43, GHRs of nonprimate mammals and birds have leucine (hydrophobic), reptile and

amphibian GHRs and teleost SLRs have glutamine (non-charged hydrophilic), and teleost GHRs have methionine (hydrophobic), all of which show strong (though not perfect) lineage-specific conservation. Considering that the substitution of R43 dramatically changes the binding specificity of the receptor (Souza et al. 1995; Yi et al. 2002), this lineage-specific conservation might indicate lineage-specific functions of these receptors. Because the lungfish and sturgeon GHRs have a glutamine at R43 as per the reptile and amphibian GHRs and the teleost SLRs, this should be interpreted as an ancestral residue.

All these ancestral reconstructions are supported by PAML analyses (Yang 1997; data not shown). Importantly, we found no amino acids unique to lungfish or sturgeon GHRs at these functional sites, despite their unique hormone receptor set.



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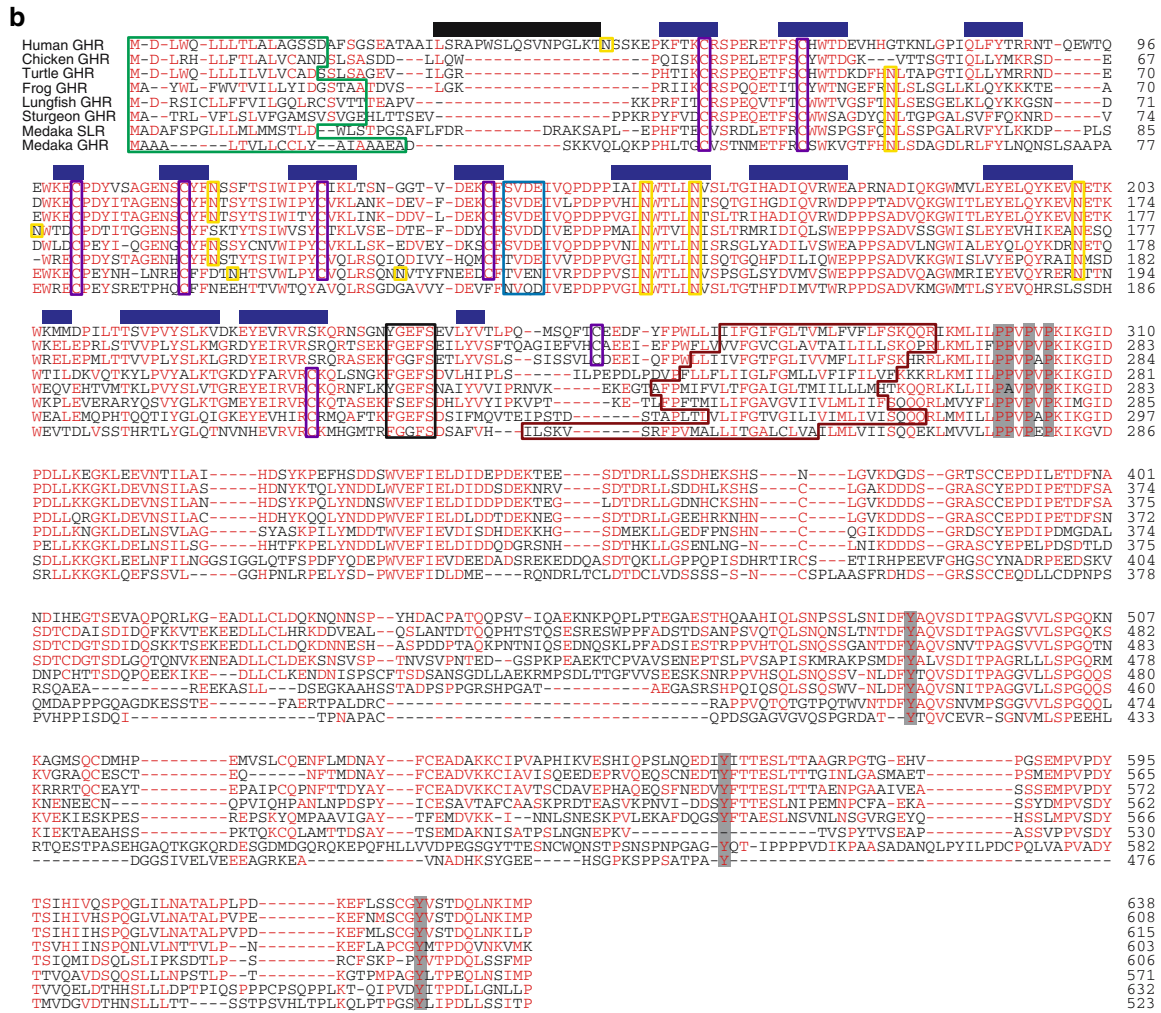


Fig. 6 continued

approximately 35% identity between GHRs of tetrapods and teleosts), the predicted secondary structures are all strikingly similar (Fig. 6a).

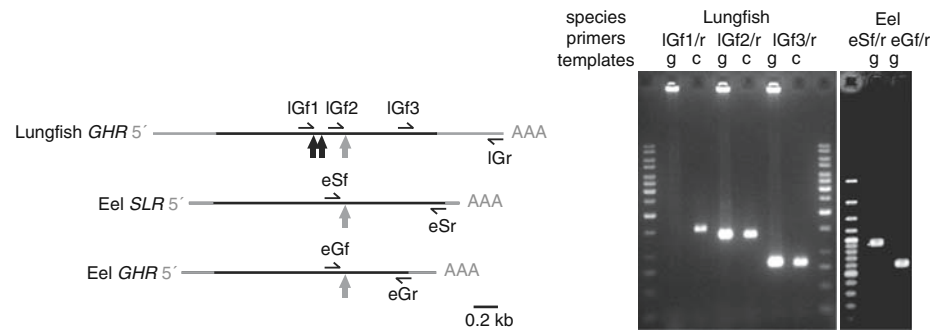
However, we note that the length of linker residues between the ECD and the transmembrane domain (TMD) appeared to differ in a lineage-specific manner. Between the bottom of the ECD and the top of the TMD (around Y230 F247), there is a linker of 16 or 17 amino acids in placental GHRs. The linker of teleost SLRs is perfectly conserved for 12 amino acids, and that of teleost GHRs is only nine amino acids (with one exception, again in the eel, of 12 amino acids). Marsupial and amphibian GHRs have relatively divergent linkers of 13–20 amino acids (longer in more derived species). We suspect that these differences are not trivial because the linker length is rather divergent as a whole (9–20 amino acids), yet is strictly conserved within each lineage. Furthermore, the length of the linker characterizes structural rigidity/flexibility of the ECD. A recent study showed that GH-mediated rotation of the dimerized receptors at an appropriate angle is necessary for its activation

(Brown et al. 2005). Lungfish and sturgeon GHRs have linkers of 14 and 12 amino acids, respectively; again similar to other teleost SLRs and amphibian and reptilian GHRs.

Taken together, our residual and structural comparisons did not reveal any unique features specific to the lungfish and sturgeon GHRs. Instead, they always share identical residues and similar structures with some or all of other GHR/SLRs. The similarities (e.g., overall sequence similarity, position of paired/unpaired cysteines, residues at R43, linker length, glycosylation sites, etc.) are more often observed with teleost SLRs and poikilothermic-tetrapod GHRs rather than with homeothermic-tetrapod and teleost GHRs.

#### Genomic Structures of the Lungfish and Sturgeon GHRs

In previous studies, orthology relationships of eel GHR/SLR to other teleost receptors remained ambiguous (Fukamachi et al. 2005; Ozaki et al. 2006). Although the



**Fig. 7** Absence of the *SLR* specific intron from lungfish/eel *GHRs* and also from eel *SLR*. Expected positions of the intron (gray arrows) and approximate positions of the primers are shown on the left diagram. Expected positions of the two conserved introns on the

lungfish *GHR* are shown by black arrows. Templates and primers used for PCRs are shown above the gel image (c, cDNA; g, genomic DNA). The same result was obtained from sturgeon (data not shown)

present study clarifies their relationships (Fig. 4), the phylogenetic position of the eel *SLR* is unexpectedly high up in the gene tree and does not conform to its species phylogeny when analyzed by both Bayesian and maximum-likelihood methods (compare the tree topologies between the *GHRs* and *SLRs* of teleosts). For further clarification of their evolutionary history, we investigated whether the extra intron specifically found in teleost *SLRs* (Fukamachi et al. 2005) is found in the eel receptors as well. We also analyzed the lungfish and sturgeon *GHRs* in this regard. Comparisons of RT-PCR and genomic PCR products, however, showed that none of these receptors have the extra intron (Fig. 7). We also found that the intron does not exist in the predicted zebrafish receptors (Fig. 2). Therefore, it is likely that the ancestral *GHR* did not have this intron and *SLRs* later acquired it after the evolutionary separation of eels, and probably Cypriniformes, from the stem lineage that leads to more derived teleosts.

## Discussion

Our present analyses on the *GHR/SLR* sequences of tetrapod and teleosts, and also the newly determined *GHR* sequences of “living fossils” revealed for the first time that (1) the *SLR* is a teleost-specific *GHR* paralogue acquired most likely through the FSGD, (2) there are multiple lineage-specific residues and structures among tetrapod *GHRs*, teleost *GHRs*, and teleost *SLRs*, and (3) the lungfish and sturgeon *GHRs* share similarities more often with teleost *SLRs* and amphibian and reptilian *GHRs* than with mammalian, avian or teleost *GHRs* (Figs. 2, 4, 5, and 6).

### Clarification of the Nomenclature of Teleost Receptors

Currently, *GHR* and *SLR* of teleosts are often referred to as *GHR2* and *GHR1*, respectively (Saera-Vila et al. 2005; Jiao

et al. 2006; Ozaki et al. 2006). This nomenclature is basically (evolutionarily) correct according to our present results, which showed that both the teleost *GHR* and *SLR* are orthologous to the mammalian *GHR* (Figs. 2 and 4). Also to be reconsidered, however, is that both *GHR1* and *GHR2* in salmonids represent *GHR* (*GHR2*), which reflects another (4R) polyploidization (Moghadam et al. 2005; Fig. 4). *GHR1* was defined as *SLR* in salmon based on functional evidence; SL binds to this receptor more strongly than GH does (Fukada et al. 2005). In order to further support this nomenclature which does not reflect the evolutionary history (Mindell and Meyer 2001), further evidence showing that *SLR* really functions as a receptor for SL would be necessary.

The clearest evidence would be provided through the isolation of mutants or knockouts for *SLR* as has been done for human, mouse and chicken *GHRs* (Agarwal et al. 1994; Zhou et al. 1997; Laron 2004). Experiments using medaka would be the most logical, because a medaka mutant for SL has already been isolated and a gene-knockout method (TILLING) has been established in this species (Fukamachi et al. 2004; Taniguchi et al. 2006). Precise agreements between the phenotypes of the *ci* mutant and a *SLR* knockout, if obtained, would clearly establish that *SLR* is a necessary and sufficient receptor for SL. Such in vivo evidence will also exclude the possibility that the in vitro binding data only reflect a less significant cross-reaction, as has been shown between GH and PRLR in human (Somers et al. 1994).

### Ancestral Receptor for SL

Even if such functional evidence would support the validity of the *SLR* nomenclature, the question would remain: which receptor(s) did SL bind to before the FSGD (Fig. 1)? The ancestral characteristics that are retained relatively frequently in teleost *SLRs* (Figs. 5 and 6) and the in vitro

binding of not only SL but also GH to the salmon SLR (Fukada et al. 2005) seem to be suggestive in this regard. Another intriguing result was recently reported for the eel: while its SLR specifically binds to GH, its GHR binds to both GH and SL (Ozaki et al. 2006; personal communication). These examples from two teleost species do not contradict each other in that one of the duplicated GHRs (i.e., salmon GHR and eel SLR) binds specifically to GH while the other (i.e., salmon SLR and eel GHR) binds to both GH and SL. Indeed, it appears that the SLR-like (i.e., probably more-ancestral) features are exceptionally retained in eel GHR (e.g., overall sequence similarity, linker length, conserved cysteines, etc.; Figs. 5 and 6). Therefore, the presumed role of GHR in the vertebrate ancestor was as a receptor for both GH and SL. After the FSGD, this dual function might have been subfunctionalized in teleosts: one became the receptor for GH and the other for SL.

This ancestral dual function (i.e., the GHSLR hypothesis) may be testable through functional studies on “living fossils” representatives of basal lineages of the fish and tetrapod lineages. However, the lungfish or sturgeon GHRs would not necessarily have retained the ancestral function precisely as it was when their lineages arose in the Devonian period, since their hormone receptor relationships may also have diverged in a lineage-specific manner during more than 400 million years of evolution. Therefore, the results obtained from such experiments could not be interpreted as strong evidence either for, or against, this hypothesis. Nevertheless, *in vitro* binding of their GHRs to both GH and SL, as is the case for the salmon SLR and eel GHR, and consequent activations of mutually different target genes (if obtained) would most parsimoniously support the GHSLR hypothesis. The species from which we cloned GHRs (*P. dolloi* and *H. dauricus*) and the species from which GH/SL sequences were reported (*P. annectens*, *A. gueldenstaedtii*, and *A. transmontanus*) are unfortunately different. In order to avoid the lineage- and species-specific artifacts (e.g., R43 of primate GHRs discussed in the “Results” section), more cloning work (or purification of the hormones from these endangered fish) would be necessary to rule out this possibility.

An alternative hypothesis for the ancestral receptor for SL would be that the common vertebrate ancestor had a novel receptor for SL that is not orthologous to the GHR/SLRs. The sequence of this putative receptor should not differ drastically from those of GHR/SLRs because SL is the closest relative of GH. Although our degenerate primers did not amplify such a sequence in any of the three ancient fish examined, an examination of their complete genome data or extensive screening, using, for example, the two-hybrid system, might reveal the existence of such a receptor(s). However, if these fish had retained such a

receptor(s) during evolution, this putative receptor would have been retained in at least some of the available tetrapod and teleost genomes but that does not seem to be the case.

#### Receptor for SLb and Future Subjects To Be Addressed

The FSGD duplicated not only *GHR* but also *GH* and *SL*. While the duplicated *GH* seems to have been lost from the genomes of medaka, fugu, or zebrafish, the duplicated *SL* is retained in zebrafish (Fig. 3) and some other teleost lineages (Zhu et al. 2004). The duplicated SLa and SLb have limited sequence similarities (<50%) and the receptor for SLb has not been investigated in any species to date. The additional polyploidization in salmonids (Moghadam et al. 2005) and a possible loss of GHR in turbot (Saera-Vila et al. 2005) makes generalizations about the overall hormone receptor relationships in teleosts difficult. These inconsistent hormone receptor sets and their distinctive expression patterns and functional diversifications (e.g., Fukada et al. 2004; Zhu et al. 2004; Fukada et al. 2005; Fukamachi et al. 2005; Saera-Vila et al. 2005; Very et al. 2005; Jiao et al. 2006; Ozaki et al. 2006; Zhu et al. 2007) would indicate that the *in vivo* roles of these orthologous genes are not necessarily identical in all teleost species. Our results aid in the clarification of the evolutionary histories of these hormones and receptors. Comparative studies with divergent species (e.g., medaka and zebrafish) using not a part but the entire hormone receptor set of this gene family, would be necessary to provide reliable clues about past and present functions of these hormones and receptors.

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